FABRICATION AND EXTRACTION OF SILVER NANOPARTICLE USING *Bacillus thuringiensis*

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CERTIFICATE

*This is to certify that the thesis entitled "**Biofabrication and extraction of silver nanoparticle using Bacillus thuringiensis**" submitted by **Mr. Ajeet Kumar** (Roll No: 412LS2039) in partial fulfilment of the requirements for the award of **Master of Science in Life Science** to the National Institute of Technology, Rourkela, is an authentic and original record of research work carried out by him under my supervision and guidance.*

To the best of my knowledge, the work incorporated in this thesis has not been submitted elsewhere for the award of any degree.

Place: Rourkela

Date: 11th May '14

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DECLARATION

To the best of my knowledge the research project report entitled "**FABRICATION AND EXTRACTION OF SILVER NANOPARTICLE USING *Bacillus thuringiensis***" reported here in its original and has been submitted to National Institute of Technology, Rourkela for partial fulfilment of the degree of Master of Science in Life Science is a bonafide record of the project work carried out by me under the supervision of Dr. Suman Jha, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. This thesis is my own work and that, to the best of my knowledge and belief, the matter and results of this thesis has not been submitted by any other research persons or any students.

I do hope, this project work will satisfy our beloved teachers. I solicit kind and favourable appreciation.

Mr. Ajeet kumar

Dedicated *to*

Respected Teachers, friends and my family

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ABBREVIATIONS

Ag- Silver

NP- Nanoparticles

($AgNO_3$)- Silver nitrate

DLS- Dynamic light scattering

ATR-FTIR- Fourier transform infrared spectroscopy

SDS- Sodium dodecyl sulphate

nm- nanometer

MIC-Minimal inhibitory concentration

mV- milli volt

FE-SEM- Field Emission Scanning electron microscope

ABSTRACT

Nanoparticles being the smallest unit of nanotechnology are playing an important role in various fields of our life now a day. There are different kinds of nanoparticles ranging from metals to non-metals and also from carbon sources. The process of their synthesis is also different. We have used green synthesis to synthesize nanoparticles i.e. bio fabricated one. Silver nanoparticles keep an important place in area due antimicrobial property, helpful in drug delivery, application in textile industry and so on. We have synthesized silver nanoparticles using bacteria *Bacillus thuringiensis* in log phase of their growth. Firstly the tolerance capacity of bacteria was checked by MIC and then its characterisation was done with the help of UV-Visible spectroscopy, ATR-FTIR, DLS, zeta potential and FE-SEM analysis. After characterisation it was extracted from bacterial media by adding 10 mM SDS which stabilizes it. Now it was run in size exclusion chromatography and again same techniques were applied to confirm the presence of nanoparticles in the stabilising media. To check the stability of synthesized silver nanoparticle it was characterised for a week using DLS and zeta potential analysis. Results focuses on the presence of nanoparticles as well as its stability in the media as tested for 7 days. These synthesized nanoparticles can be further be lyophilized and can be modified according to need for the welfare of common mass.

1. INTRODUCTION

1.1 Brief introduction of silver nanoparticle

Nanoparticles are ultrafine particles having one of its dimensions in the range of 1-100 nm. According to the International Organization for Standardization (ISO), a nanoparticle is a discrete nano-object where all three Cartesian dimensions are less than 100 nm. Due to this reduction in size of metal it adopted some different properties from that of corresponding macromolecule. Some of the silent features of nanoparticles include its high mobility in free state, enormous specific surface area and the tendency to express quantum effects. Different metals are being synthesized into its corresponding nanoparticles. On the basis of characteristic features a nanoparticle can be differentiated accordingly. Some of them are positively charged while some are negatively while others are neutrals. Nanoparticles may be either magnetic or nonmagnetic types and they can also be classified as organic (mainly carbon) and inorganic (silver nanoparticles, gold nanoparticles etc.).

Synthesis of nanoprticles can be done by various processes including physical, chemical and biological processes. Biological way for the synthesis of nanoprticles is also known as “green synthesis”. Green synthesis is preferred over the rest because chemical method requires both strong and weak reducing agents and protective agents which are toxic to environment, but biological method is non toxic and is good in regard to the environment. It has been also found to be of low production rate in chemical method [1],[2],[3],[4]. Biological methods for synthesis of nanoparticle employ the use of biological agents like bacteria, fungi, yeast, actinomyces and plants. Biological agents secrete a large amount of enzymes which are capable of hydrolysing metals and thus bring about the enzymatic reduction of metal ion [5]. Nanoparticles synthesized from bacteria are preferred over plants or other biological agents as it contains no flavenoids than that of plants contains, which

creates problem for appropriate quantification of nanoparticles. In bacterially synthesized mode the culture can be led for batch culture for high yield of nanoparticle.

Silver has the highest electrical and thermal conductivity among metals, making it a popular material for electrical contacts and an additive for conducting adhesive. More recently, silver nanoparticle have been shown to locally amplify light by 10–100 times, leading to surface-enhanced Raman scattering (SERS), with enhancement factors on the order of 10^6–10^8. There occurs a strong interaction of the silver nanoparticle with light because the conduction electrons on the silver nanoparticle surface undergo a collective oscillation when excited by light at specific wavelengths known as a surface plasmon resonance (SPR), this oscillation results in unusually strong scattering and absorption properties. Thus it can be stated that silver nanoparticle can have effective extinction (scattering and absorption) cross sections up to ten times larger than their physical cross section [6].

Silver nanoparticles synthesized from *Bacillus thuringiensis* is different from other biological approaches, this is because of the tolerance capacity of this bacteria is high than that of certain other bacterial groups like *B. substilis, B. vulgaris* and also from *E. coli*. We have focused on nanoparticles synthesis from exponential growth of bacteria i.e. bacterial cells in a log phase and are not dying. Silver nanoprticles thus synthesized have the antimicrobial property and also it can be expected to be its wide application in the areas like bioremediation, therapeutics and biological interaction of these nanoprticles with different molecules inside body like DNA, protein or enzymes. In drug delivery system it has a wide application and thus a good carrier to those specific areas where drug can’t reach and lay down their effect.

1.2 APPLICATIONs OF Ag NP

1) Silver nanoparticle showed antifungal effects on fungi tested with low haemolytic effects against human erythrocytes.
2) In plaque reduction assay: nanoprticles (10-80 nm, with or without polysaccharide coating), or silver nitrate ($AgNO_3$) at concentrations of 100, 50, 25, and 12.5 ug/mL were evaluated for efficacy using a plaque reduction assay. Both Ag-PS-25 (polysaccharide-coated, 25 nm) and Ag-NP-55 (non-coated, 55 nm) exhibited a significant (PB 0.05) dose-dependent effect of test compound concentration on the mean number of plaque forming units (PFU) [7].
3) Silver nanoparticle has antimicrobial activity.
4) It has also antifungal property.
5) Biologically synthesized silver nanoprticles have many applications including in textile industry.
6) Applications like coatings for solar energy absorption and intercalation material for electrical batteries, as optical receptors, as catalysts in chemical reactions, for boil labelling, and as antimicrobials due to the antibacterial activity.
7) Potential application of silver nanoparticle like diagnostic, biomedical, optical imaging, biological implants (like heart valves) and medical application like wound dressings, contraceptive devices, surgical instruments and bone prosthesis.

2 LITERATURE REVIEW

Silver nanoparticle is particles having one of its dimensions in the range of 1-100 nm. It can be synthesized by either bottom-up or from top-down approach. Nanoparticles can be synthesized with the help of physical, chemical and biological processes. Silica nanoparticles along with other nanoparticles are being synthesized with the help of physical and chemical methods [8]. Biological way to synthesize nanoparticles includes the use of bacteria, plants, fungi, yeast, actinomyces [9],[10],[11]. Not every bacterium can synthesize nanoparticle and also the synthesis depends on the minimal inhibitory concentration of metal ion that a bacteria can tolerate, called as "threshold value" [12]. Silver nanoparticles have been successfully synthesized from gram negative bacteria like *E.coli*. Silver nanoparticles have a characteristic feature of absorbing light at a wavelength of 420 nm [12]. Silver nanoparticles can also be synthesize from other strain of bacillus bacteria like *Bacillus licheniformis* [13]. Silver nanoparticle exhibit the property of light scattering and surface Plasmon resonance effect [14]. The dielectric constant of the environment around the silver nanoparticle also plays important role in its alteration to optical properties [15]. A shape of nanoparticles sometime also determines its optical properties. Nanobars and nanorices have different values of absorbance of light spectra. It has been reported that with increase in size of nanoparticle, the peak shifts to a higher wavelength [16].

The exact mechanism of silver nanoparticle synthesis by bacteria is not completely understood, but it seems that they use enzymes for the synthesis of nanoparticles. Mechanism widely accepted is the activity of enzyme "nitrate reductase" which is helpful in silver nanoparticle synthesis (Fig. 1). During the catalysis, nitrate is converted to nitrite, and an electron will be shuttled to the incoming silver ions. This has been excellently described in the organism *B. licheniformis*. *B. licheniformis* is known to secrete the cofactor NADH and

NADH-dependent enzymes, especially nitrate reductase, that might be responsible for the bioreduction of Ag^{+} to Ag^{o} and the subsequent formation of silver nanoparticles [17],[18].

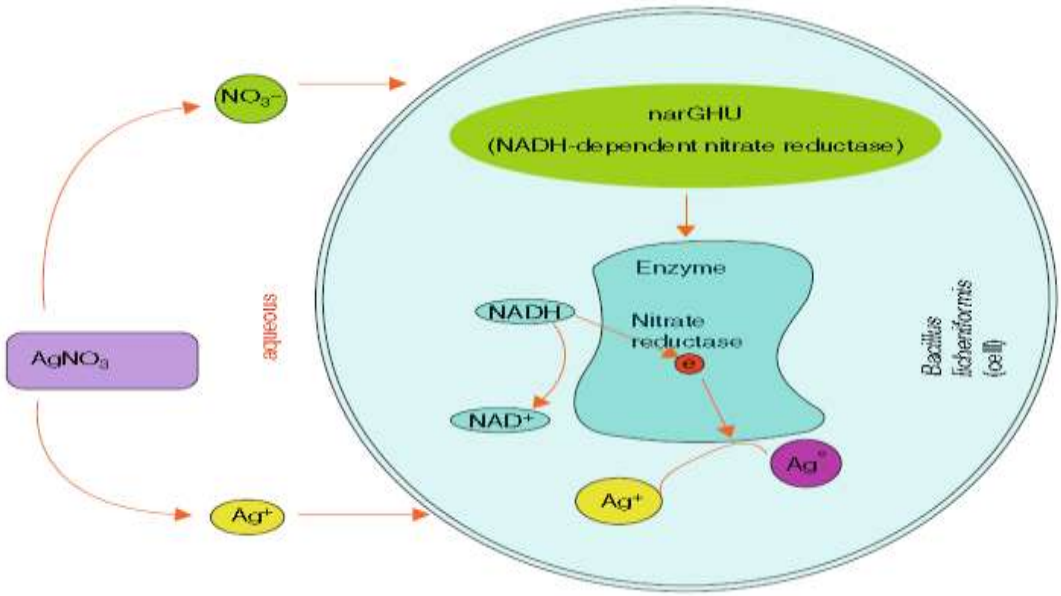

Fig. 1 Mechanism for silver nanoparticle synthesis from bacteria (adopted from Kalimuthu et al. (2008).

Table 1. Nanoparticles synthesized from different bacteria.

S.No.	Organism	Size of nanoparticle(nm)	Author
1	*Pseudomonas stutzeri AG*	259-200	200 Tanja et al. (1999)
2	*Lactobacillus Strains*	500	Nair and Pradeep (2002)
3	*Bacillus megaterium*	46.9	Fu et al. (1999)
4	*Klebsiella pneumonia* (culture supernatant)	50	Ahmad et al. (2007)
5	*Bacillus licheniformis*	50	Kalimuthu et al. (2008)

6	*Bacilluslicheniformis* (culture supernatant)	50	Kalishwaralal et al. (2008)
7	*Corynebacterium sp*	10-15	Zhang et al. (2005)
8	*Bacillus subtilis* (culture supernatant)	5-60	Saifuddin et al. (2009)
9	*Geobacter sulfurreducens*	200	Law et al. (2008)
10	*Morganella sp*	20-50	Parikh et al. (2008)
11	*Bacillus subtilis*	5–60	Saifuddin et al. (2009)
12	*Proteus mirabilis*	10–20	Samadi et al. (2009)
13	*Bacillus sp.*	5–15	Pugazhenthiran et al. (2009)
14	*Staphylococcus aureus*	1–100	Nanda and Saravanan.(2009)

Antibacterial property of silver nanoparticle provides it potent application against different bacterial cells. The entire mechanism can be described as follows:

(1) Binding of silver ion to negatively charged DNA(since prokaryotes don't contain histones) thus making DNA to lose its structure and also inhibiting the process of replication.

(2) By binding with thiol containing proteins it inhibits the function of protein.

(3) Induction of reactive oxygen species synthesis leading to the formation of highly reactive radicals that destroy the cell.

Table 2 Mechanism of antimicrobial effect of silver nanoparticle

1) Cell death due to coupling of oxidative phosphorylation
2) Cell death due to induction of free redical formation.
3) Interferance with respiratory chainat cyt. C level.

4) Interfare with components of microbial ETS.
5) Interaction with protein thiol group and membrane enzyme.
6) Interaction with phosphate and sulphur containing compounds like DNA
7) Inhibition of certain enzymes like NADH dehydrogenase II in respiratory chain system.

The synthesis of nanoparticle from extracellular synthesis from bacteria we have focused on synthesis occurring in the exponential phase i.e. log phase in contrast to stationary phase[19]. The major application of this nanoparticle synthesis is that it can be lyophilized as well as stabilized in a co-stablizer and can be used in different purposes including therapeutics. Stabilizers add a specific charge to nanoparticle, resulting in prevention of agglomeration of nanoparticle. Different types of stabilizers are used including starch, polyethylene glycol, SDS, formic acid, sodium meta hexa phosphate [20],[21],[22].

From the proposed study we can use this synthesized nanoparticle directly as therapeutics as the bacteria *Bacillus thuringiensisis* is nontoxic to human and also is a good example of recombinant vector carrying bacteria indicating that it can be used as a way to make the drug delivery process more efficient.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 CHEMICAL REQUIRED

Nutrient broth (Himedia), Silver nitrate ($AgNO_3$) (Sigma Aldrich), Tannic acid (Himedia), gluteraldehyde (Merk), Sephadex G-100 (Sigma), Sodium dodeyl sulphate SDS.

3.1.2 GLASS WARES

All glass wares used (conical flask, beaker, measuring cylinder, test tubes, duran tubes, petri dishes, glass column-were purchased from Borosil (India) and Rivera (Germany).

3.1.3 STRAIN USED

Bacillus thrunigenesis was purchased from IMTECH Chandigarh.

3.2 METHODS

Mainly two processes were done i.e. synthesis of nanoparticles and extraction of nanoparticle. In the synthesis phase firstly the tolerance capacity of bacteria against the metal ion was detected by minimal inhibitory concentration test (MIC), followed by its synthesis on large scale and then analysis by UV-Visible spectroscopy, FTIR, DLS and zeta potential analysis. Its morphology and size was further analyzed by SEM image. After the synthesis of nanoparticle it was extracted with the help of size exclusion chromatography using Sephadex G 100 as a stationary phase and SDS as a mobile phase through which the eluent can pass through. After the extraction of nanoparticle it was again characterised by UV-Visible spectroscopy, FTIR, DLS and zeta potential analysis. The entire process can be explained in detailed as follows:

3.2.1 MIC (Minimal Inhibitory Concentration) test

MIC was performed to check the minimum concentration of silver metal ions at which the growth of bacterial can be inhibited. It is important to perform ~~it~~ to know the tolerance of bacteria to resist silver metal ion concentration. *B. thuringiensis* was grown in nutrient broth media and during its log phase (3-4 hrs after incubation) metal ion of different concentrations was added to it on 96 walled micro-titre plates and was incubated for 24 hrs in plate reader maintaining temperature of 37^0C and simultaneously absorbance was taken. After knowing the MIC value culture was grown on a large scale and was characterised.

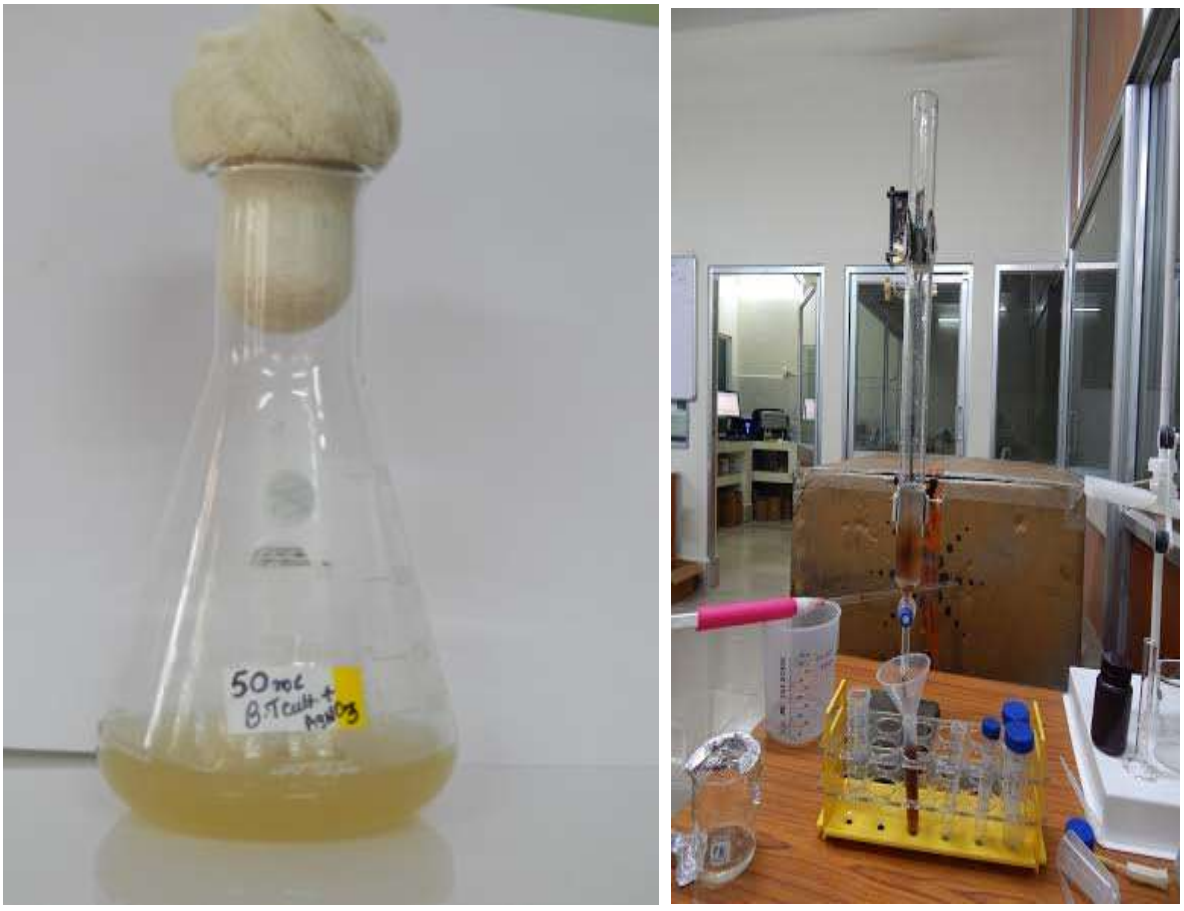

Fig. 2 (a) *Bacillus thuringiensis* containing silver ion, (b) Size exclusion chromatography of Ag NP

3.2.2 UV-Visible spectroscopic analysis

To check the surface Plasmon resonance property of nanoparticle synthesized from bacteria it is necessary to go for its UV-Visible spectrum analysis which reveals the specific type of nanoparticle absorbing a specific wavelength of light. This property can distinguish silver nanoparticle from others and can also state whether it is silver or not present in the solution. UV-Visible spectroscopy works on the principle of light absorption depending on the concentration of particles in the solution. Silver nanoparticle has a unique property of surface Plasmon resonance. Here the electron on the metal surface has its own frequency due to oscillation against the electro positive nuclei. In the case of nanoprticles SPR is known as localized surface Plasmon resonance.

3.2.3 ATR- FTIR-Analysis

The samples were then characterised by FTIR. In FTIR the vibration of chemical bonds can be measured because chemical bonds can absorb infrared energy at specific frequencies or wavelength. The basic structure of the compound can be determined by spectral location of their IR absorption. It can also state about other molecules being associated on the surface of nanoparticle and thus predicts possible interaction of nanoparticles with other molecules.

3.2.4 DLS (Dynamic light Scattering) analysis

Particles of different sizes can scatter light to different angles on the basis of their difference in size. The scattering of light is based on particle size, smaller particles can scatter light strongly while increase in particle size leads to less scattering. Samples were analyzed after synthesis of nanoparticles and it was again performed when the nanoparticles were extracted from bacterial media.

3.2.5 ZETA POTENTIAL Analysis

It mainly determines the net charge on the surface of nanoparticle. Nanoparticles have a surface charge that attracts a thin layer of ions of opposite charge to the nanoparticle surface. This double layer of ions travels with the nanoparticle as it diffuses throughout the solution. The electric potential at the boundary of the double layer is known as the Zeta potential of the particles.

3.2.6 FE-SEM Analysis

After the synthesis, nanoparticles were analysed under scanning electron microscope which gives a clear image to the synthesized nanoparticle. It reveals the morphological features of nanoparticles.

After the synthesis it was necessary to extract nanoparticles from the media and also to be stored in a media in which it could be stabilized. To extract the nanoparticles, it was first mixed with SDS and then was run in size exclusion chromatography where the stationary phase was sephadex G-100 and the mobile phase contains 10 mM SDS in deionized water. The flow rate was 1 mL/minute [23, 24]. The eluents were collected in different falcons and were again characterised by UV-Visible spectroscopy, ATR-FTIR, DLS and Zeta potential analysis.

4 RESULTS AND DISCUSSION

4.1.1 MIC RESULT

For the synthesis of silver nanoparticles it was first checked the tolerance capacity of bacteria against a given concentration of silver ion concentration. This is the intrinsic property of a bacterium and also known as “threshold value” for that bacterium. Silver ion concentration from 5mM to 0.009mM was given to the bacteria growing in nutrient broth media. The bacterial populations were in their lag phase. After 24 hours of incubation the UV-Visible absorption reading was taken in plate reader. From the reading it was confirmed that at the concentration of 0.15mM absorption increases which was not shown to previously added concentration. Thus it was confirmed that it is the minimal concentration at which bacteria can tolerate metal ion concentration and can grow.

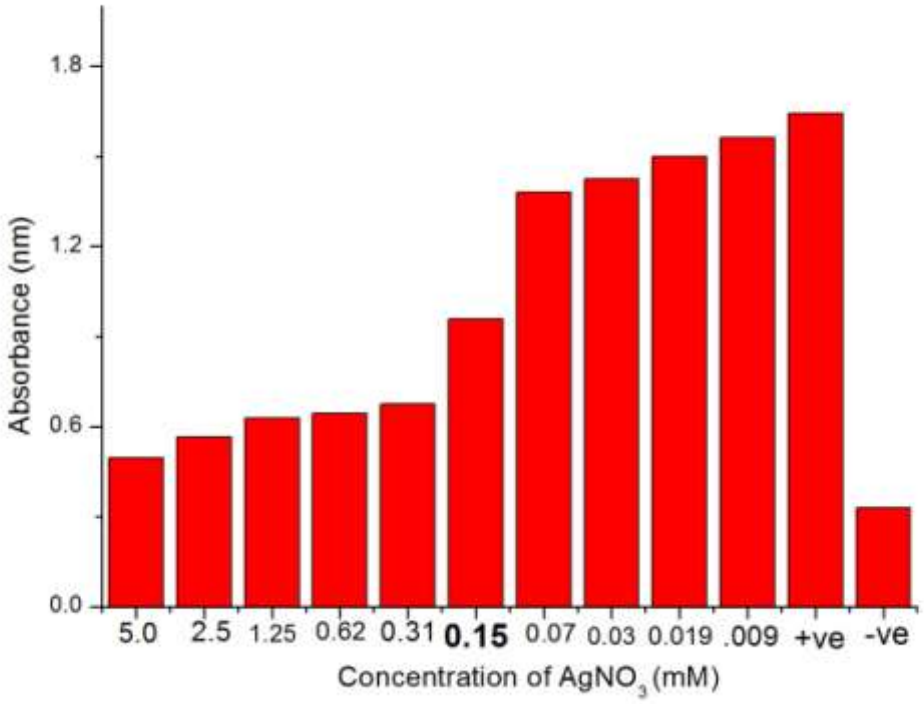

Fig. 3 MIC analysis of *Bacillus thuriengesis* against silver ion concentration after 24 hours.

Thus from the above bar diagram we can conclude that bacteria are resistant to silver ion concentration of 0.15mM and is further led for the synthesis of corresponding nanoparticle.

After having the proper information regarding the required concentration of metal ion concentration for nanoparticle synthesis it is led for production on large scale (200 ml).After the synthesis, it was characterised by different t techniques for the presence of nanoparticle. The main important techniques implemented were UV-visible spectral analysis, Fourier Transform Infrared (FTIR) analysis, Dynamic Light Scattering (DLS) analysis, Zeta potential analysis and finally Field Emission -Scanning Electron Microscopy (FE-SEM) analysis. These characterisation results are described as follows:

4.1.2 UV-Visible Spectral analysis:

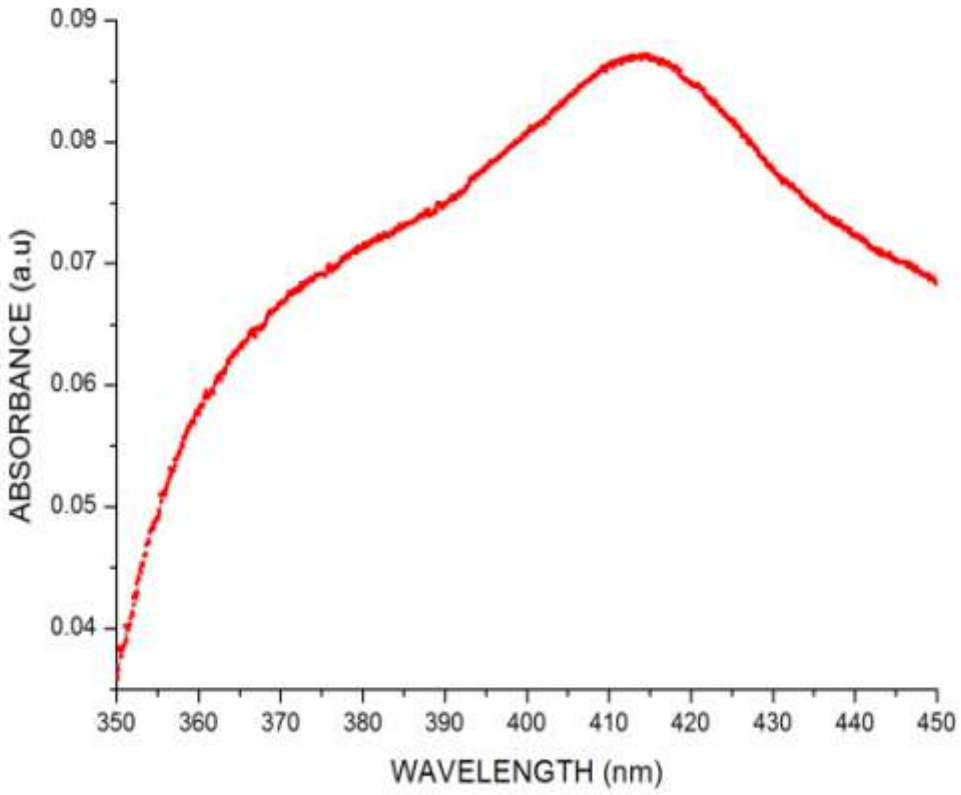

Fig 4 UV-Visible spectroscopic analysis of synthesized nanoparticle

The sample after the production of silver nanoparticle was characterised under UV-Vis spectroscope at wavelength 350nm to 450nm (fig.4). It has been studied that the silver nanoparticle absorbs maximum light at the wavelength 420nm[12 ,25]. From the graph we found the absorbance peak at 415nm. The presence of sharp peak confirms the homogeneity of silver nanoparticles.

4.1.3 ATR-FTIR Analysis:

Since every molecule has the property to absorb the light in IR region and this absorption makes the vibration of the bonds present in the molecules. Every molecule has a specific vibration which can be detected by using FTIR.

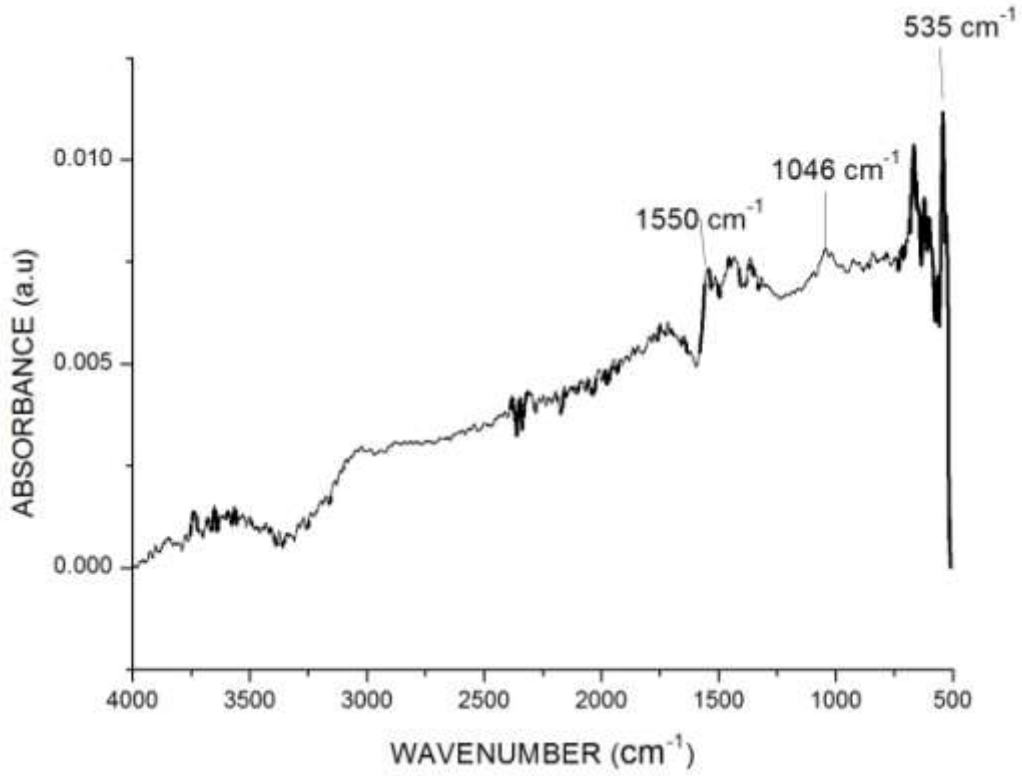

Fig. 5 ATR-FTIR Analysis of synthesized silver nanoparticle.

There are different stretches present in the ATR-FTIR graph. Silver nanoparticles show characteristic peaks between 500-600 cm^{-1}. In figure 5, a peak is observed at 535cm^{-1}, which is due to presence of silver nanoparticles. The peaks found at 1046cm^{-1} and 1550cm^{-1} are due to phosphate ions and for polyols, which are actually carbonyls and play important role in reducing silver ion. There are also some different stretches which shows the presence of different moieties that make interaction with the synthesized nanoparticle.

4.1.4 DLS Analysis:

To determine the particle size we perform dynamic light scattering technique. Here the radius of the particle is responsible for light scattering. Particle with greater radius diffract the light less than that of a smaller radius particles.

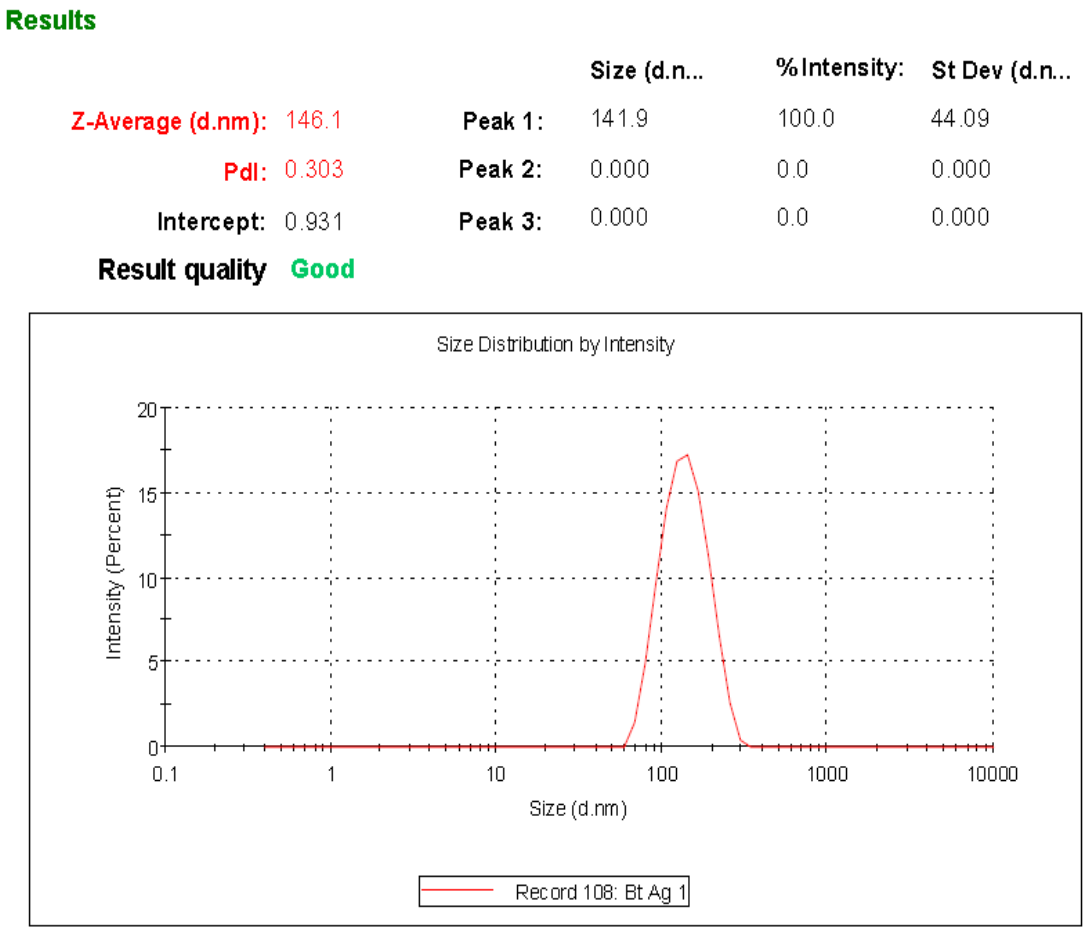

Fig. 6. DLS analysis of synthesized silver nanoparticle.

The fig.6 shows the DLS analysis of silver nanoparticles. The average particle size determined by DLS was found to be 146.1 nm. The presence of single peak confirms that particles are in mono dispersed form.

4.1.5 ZETA POTENTIAL analysis:

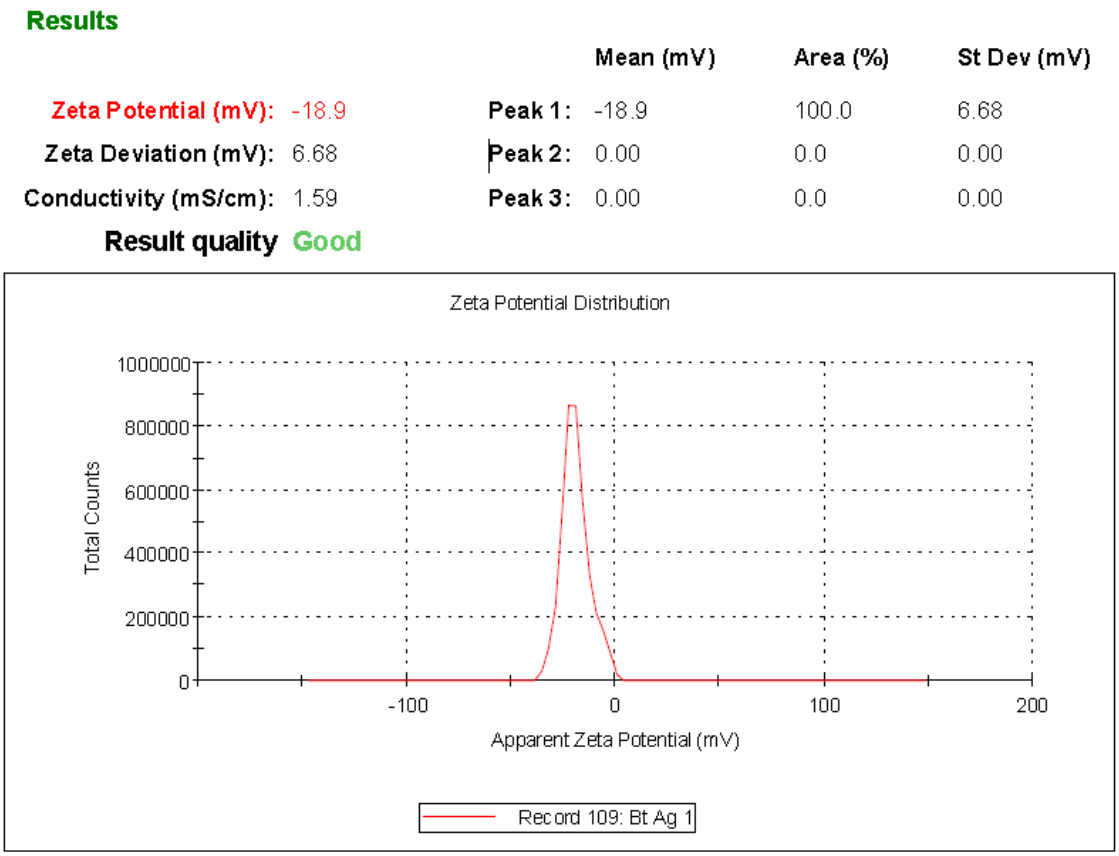

Fig. 7: Zeta potential value of synthesized silver nanoparticle.

The observed zeta potential for the synthesized nanoparticle was found to be -18.9 mV. It indicates that the surface of the synthesized silver nanoparticle has negative charge. The observed value also points towards the stability of nanoparticle.

4.1.6 FE-SEM analysis

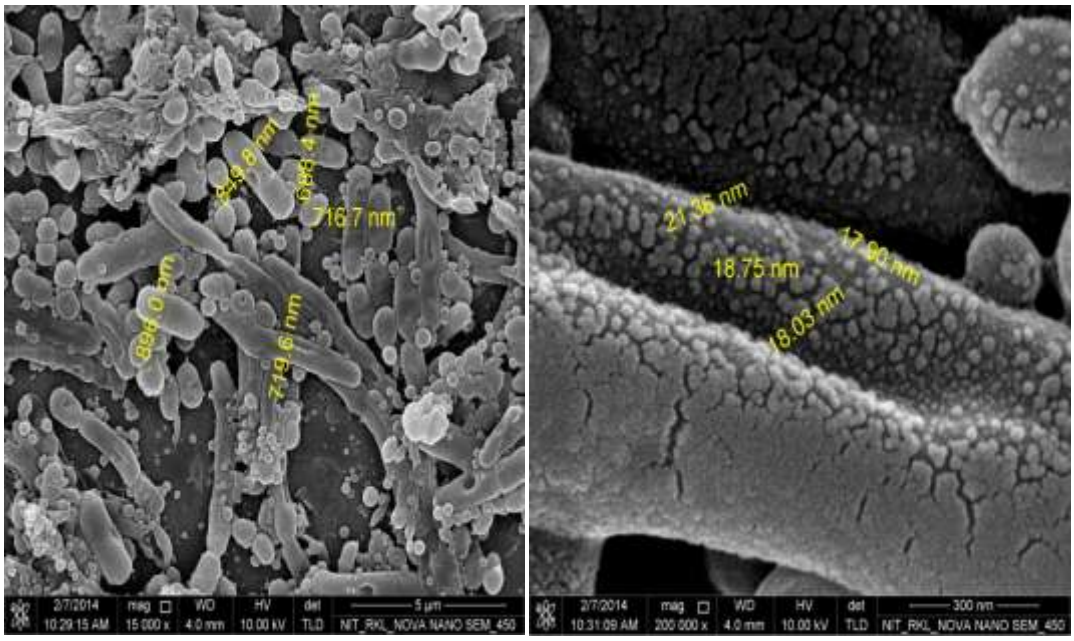

Fig.8 (a) *Bacillus thuringiensis* under, FE- SEM Analysis

8(b) Silver nanoparticle over the surface of *Bacillus thuringiensis*

From the SEM analysis we can conclude that the synthesized nanoparticle is of circular form with different sizes ranging from 17 nm to 21 nm. They are present on the surface of bacteria indicating their synthesis to be extracellular. Also in fig.8 (a) it is observed the size of bacteria mainly the diameter with a value of 716 to 896 nm. There is a presence of other molecules in this figure; it may be nanoparticle of bigger size in the media.

4.2 Extraction of synthesized silver nanoparticles

After the synthesis of silver nanoparticle it was extracted from the bacterial media by size exclusion chromatography using sephadex G-100 as a stationary phase while the mobile phase was 10M SDS. The flow rate was kept as 1ml per minute. Different elutions were isolated at a regular interval and marked as elution 1, 2, 3, 4, 5 and 6. Now these elutions were characterised by, FTIR analysis, DLS analysis and zeta potential analysis.

4.2.1 UV-Visible spectroscopic analysis:

The absorbance of all the eluents under UV-Visible analysis were performed to check the presence of silver nanoparticle.

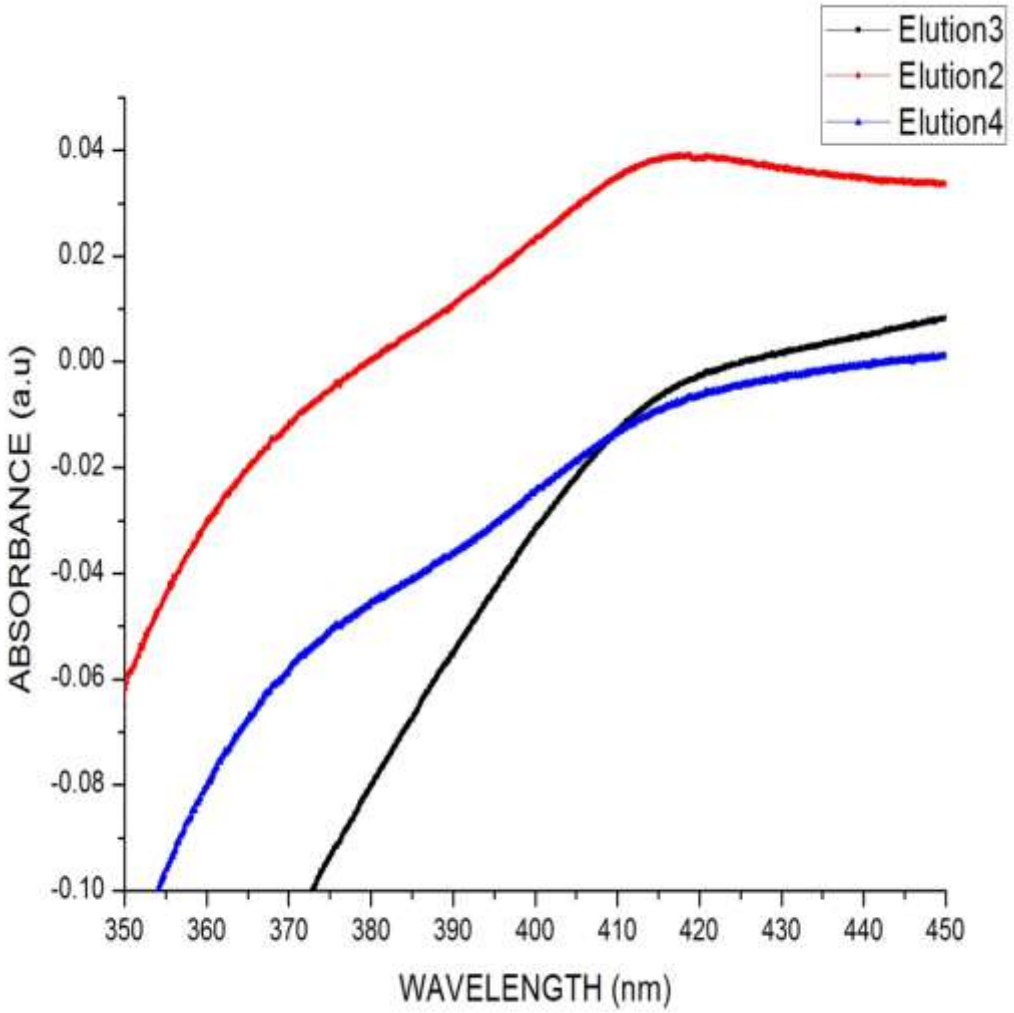

Fig 9 UV-Visible spectroscopic analysis of different elution containing silver nanoparticle

From the graph we can conclude that elution 2, 3, 4 were absorbing light at a wavelength near 420 nm. The absorbance of elution 2 attends a maximum value near 0.03 followed by elution 3 and elution 4.Since the characteristic feature of silver nanoparticle is to absorb the light

maximum at a wavelength of 420 nm which reveals the presence of silver nanoparticle. Thus these elutions provides the proof of the presence of silver nanoparticle.

4.2.2 ATR-FTIR analysis

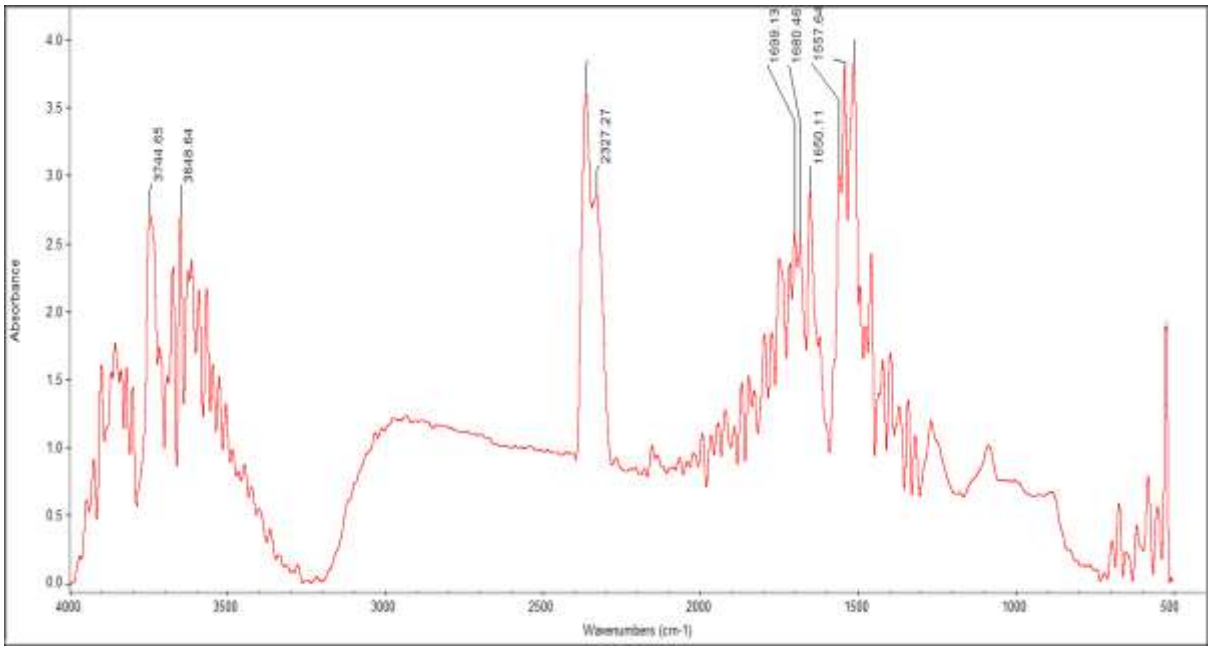

Fig. 10 FTIR analysis of extracted nanoparticles (after chromatography)

From the ATR-FTIR analysis it is confirmed that there is a presence of silver nanoparticle since a sharp peak is obtained at 538 cm^{-1}. From the literature it is confirmed that silver nanoparticle got its peak in the range of 500 to 600 cm^{-1} .There are certain other peaks present near 1500 to 1600 cm^{-1}.This shows amide II vibration this may be due to interaction of certain proteins to the nanoparticle. Another peak is obtained near 2327 cm^{-1}.This may be due to the presence of certain carboxylic acid c-o stretch.

4.2.3 DLS Analysis

To determine the particle size of synthesized nanoparticle in elutions 2, 3, 4, we perform DLS analysis. These were done at a regular interval of days and from the data we can conclude about the relative changes in particle size and the stability of nanoparticle synthesized.

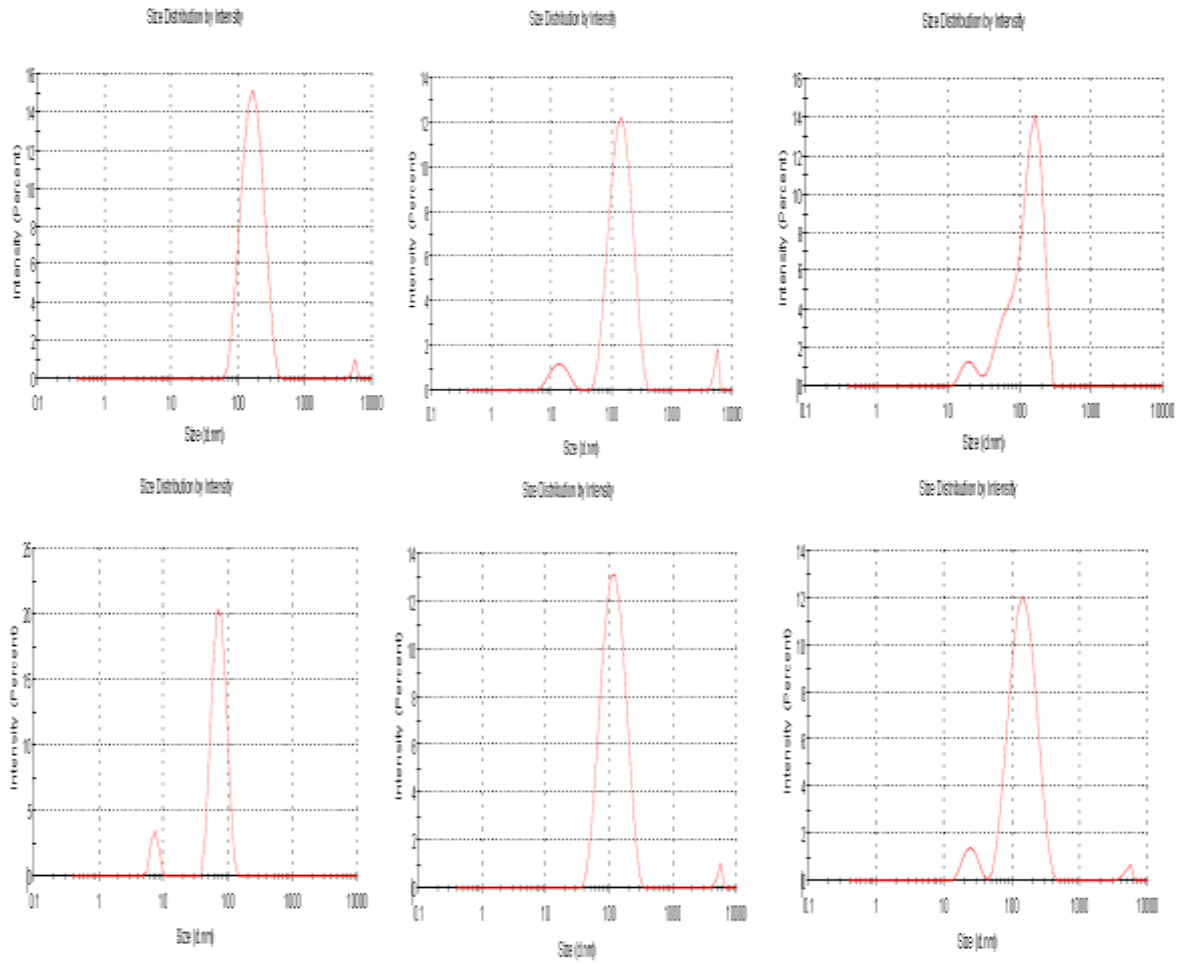

Fig.11 DLS analysis of extracted nanoparticle after elution (3rd above all and 7th day below)

In the figure 11 the DLS of elutions are having an average size of 120nm. Elution 2 are shown in figure on the top left. The average size is 164nm and after a week its size reaches to 149.3nm. Graph in the middle represents the data of elution 3 and the average size on 1st day is 159.1nm and the figure below it indicates its size 123nm after a week. In the right top the

average size of silver nanoparticle in elution 4 is 177.3nm and it reaches to 154.7 after a week as shown in figure right bottom. It can be concluded from the above data that nanoparticles synthesized retains its size in the stabilising media.

4.2.4 Zeta potential analysis

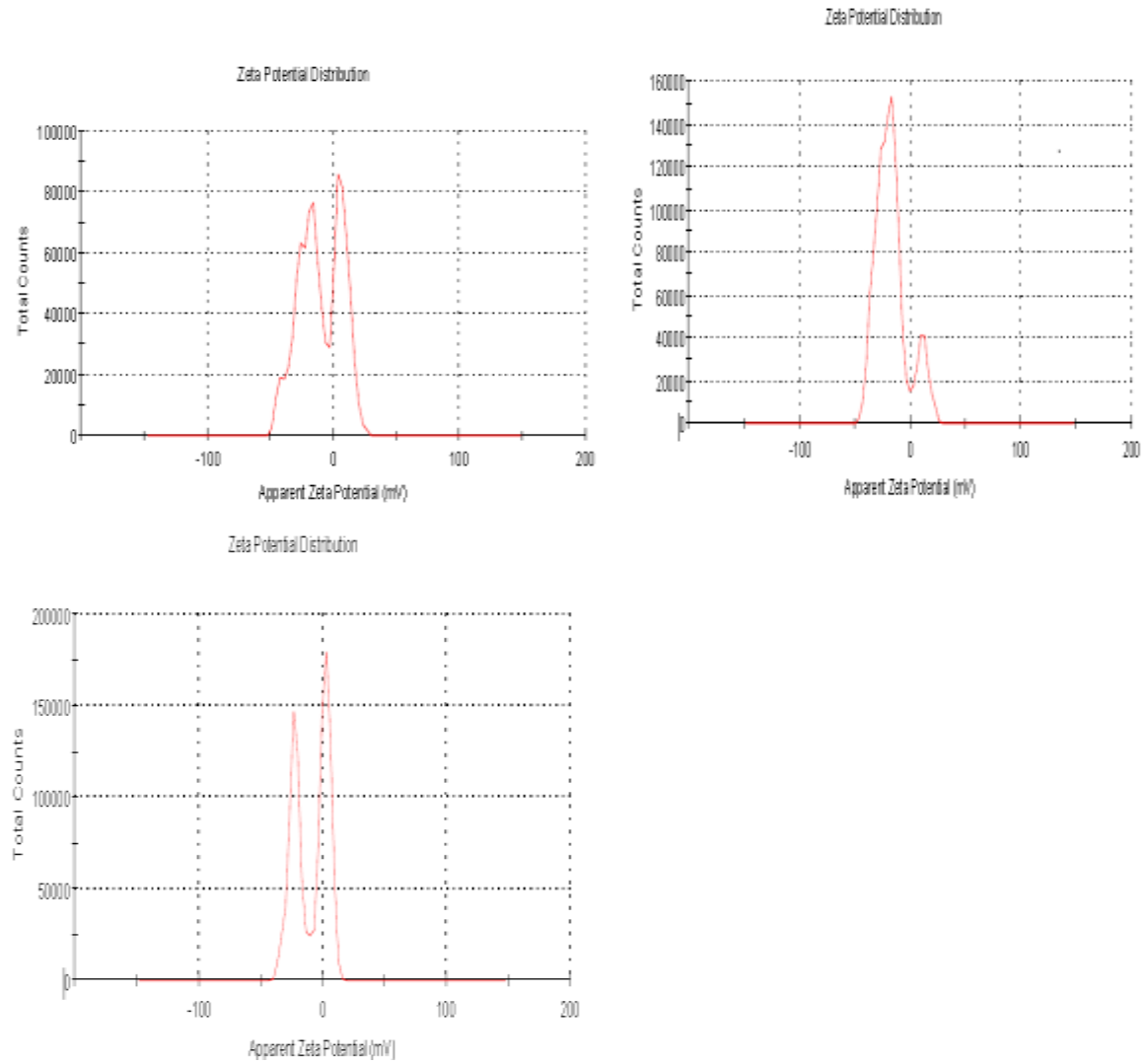

Fig.12 Zeta potential of extracted silver nanoparticle top right for 1st day, top left for 3rd day, and bottom for 7th day.

From the above figure it is clear that the extracted silver nanoparticles are of having a net negative potential and this value lies in the range of -18 to -24 mV, which indicates nanoparticles are quite stable during the storage period in stabilising media i.e. 10mM SDS. It

can be seen by the graph that the synthesized nanoparticles are going more stabilized as day are increasing by increasing its zeta potential.

5. CONCLUSION

From the work performed, it can be concluded that silver nanoparticles synthesized from *Bacillus thuringiensis* were spherical in shape. The average size varies from 130± 10 nm. Silver nanoparticles presence was characterised using UV-Visible spectroscope, ATR-FTIR, DLS, zeta potential and FE-SEM analysis. It is also concluded that the nanoparticle size is further reduced when it is extracted from the colloidal solution after fabrication using 10 mM SDS. This may have occurred due to the removal of capping agents after extraction. Exracted nanoparticle is found to be stable in the stabilising media for next 7 days at 4 ^{o}C.

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Thank You!